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Knockdown of FOXM1 suppresses cell growth and metastasis in human laryngeal cancer *via* the AKT signaling pathway

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Abstract. – OBJECTIVE: This study aims to investigate the potential regulatory effect of forkhead box M1 (FOXM1) on laryngeal carcinoma (LC) and the underlying mechanisms.

PATIENTS AND METHODS: Tumor tissues were obtained from 80 patients diagnosed with LC in our hospital. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot analysis were used to detect the expression levels of FOXM1 in LC tissues and cell lines. Transfection of small interfering RNA (siRNA) was conducted to knockdown the expression level of FOXM1. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay and cell colony assay were conducted to measure the changes in cell proliferation capacity influenced by FOXM1. Finally, invasion and migration ability was evaluated by the transwell assay.

RESULTS: FOXM1 was found upregulated in LC tissues and cells. Transfection of FOXM1 siRNA in LC cells successfully inhibited the expression of FOXM1. The knockdown of FOXM1 resulted in reduced proliferation, invasion, and migration of LC cells. Further studies indicated that the knockdown of FOXM1 suppressed the ratio of p-AKT/AKT. Besides, the impaired proliferation, invasion, and migration of LC cells induced by FOXM1 knockdown could be counteracted by application of the AKT activator Sc79.

CONCLUSIONS: The present work demonstrated that the knockdown of FOXM1 suppressed the proliferation, invasion, and migration of LC cells by the AKT signaling pathway.

Key Words:
Laryngeal carcinoma (LC), Forkhead box M1 (FOXM1), AKT.

Introduction

Laryngeal cancer (LC) is one of the most common malignant tumors in the head and neck. Laryngeal squamous cell carcinoma is the major pathological type of LC[1]. It is reported that there were 177,422 new cases of LC around the world in 2018, accounting for 1% of all the cases of malignant tumors. Among them, 94,771 people died of LC, seriously threatening the human health[2]. In particular, LC mainly affects males than females with the incidence of 5.8/100,000 and 1.2/100,000, respectively[3]. Meanwhile, smoking, drinking, and human papillomavirus infection are the risk factors for the occurrence and development of LC. Currently, therapeutic methods for LC are dominated by surgery, combined with a comprehensive treatment of radiotherapy and chemotherapy. However, the 5-year survival rate after the operation has not been improved yet[4]. Forkhead box M1 (FOXM1) is a member of the forkhead transcription factor family, which consists of more than 100 members at present. Its functions involve embryonic development, cell cycle regulation, energy metabolism, immunoregulation, and other physiological processes[5,6]. Located in chromosome 12p13.3 in the human body, the FOXM1 gene exerts a vital role in normal cell generation cycle[7]. FOXM1 is highly expressed in the tumor tissues, such as non-small cell lung cancer, breast cancer, gastric cancer, liver cancer, cervical cancer, ovarian cancer, and pancreatic cancer[8-14]. The high expression of FOXM1 is closely associated with tumorigenesis, indicating that FOXM1 might become a potential new therapeutic target of tumors. At present, FOXM1 involvement during the occurrence and development of LC is rarely reported. We collected 80 specimens of LC tissues and corresponding adjacent tissues in our hospital, and detected the expression levels of FOXM1. Furthermore, the role of FOXM1 in cell proliferation and metastasis was analyzed, as well as the potential signaling

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pathway involved in. The aim of our study was to reveal the molecular mechanism of the development of LC and to provide a new theoretical basis for the diagnosis and treatment of LC.

Patients and Methods

Tissues

80 cases of LC tissues and matched adjacent normal tissues were surgically removed from patients admitted in the Second Affiliated Hospital of Xi'an Jiaotong University. All tissues were confirmed by pathological diagnosis. None of the enrolled patients were treated with radiotherapy, chemotherapy, or other immunological treatment. This research was approved by the Ethics Committee of Second Affiliated Hospital of Xi'an Jiaotong University. Signed written informed consents were obtained from all participants before the study.

Cell and Culture

Hep-2 cells and HaCaT cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) at 37°C in a humid atmosphere with 5% CO_2. When the cell growth reached 80-90%, the original medium was removed, and cells were washed twice with phosphate-buffered saline (PBS). Subsequently, cell passage was conducted in a 0.25% trypsin solution, terminated by applying the complete medium and centrifuged at 1000 rpm for 5 min. The precipitant in the centrifuge tube was suspended in fresh medium, and inoculated in a 6-cm culture dish at 1:3.

Cell Transfection

For cell interference, FOXM1 si-RNA was synthesized and applied in the study. Briefly, the cells were seeded into six-well plates. After cell culture for 24 h, 100 nM siRNA were transfected into Hep-2 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's descriptions. Next, the cells were harvested for 48 h or 72 h after transfection for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) or Western analysis, respectively. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, colony formation assay, invasion, and migration assay were performed after transfection with FOXM1 siRNA for 48 h.

QRT-PCR Analysis

The cells were lysed with TRIzol (Invitrogen, Carlsbad, CA, USA) on ice for 5 min; then, they were added into RNase-free Eppendorf (EP) tubes. 1000 ng of ribonucleic acid (RNA) was reversely transcribed into complementary deoxyribonucleic acid (cDNA) on a polymerase chain reaction (PCR) amplification instrument after the concentration and purity of RNA were determined using NanoDrop. Later, 1 μL of cDNA was taken for qRT-PCR on Step OnePlus instrument (Applied Biosystems, Foster City, CA, USA) following reaction conditions: pre-denaturation at 95°C for 30 s, and then 95°C for 5 s, and 60°C for 30 s for 40 cycles, followed by dissociation stage. With glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference, the relative quantity value was applied for analysis based on the Ct value of samples in each reaction tube. The $2^{-\Delta\Delta Ct}$ method was utilized to calculate the fold change relative to that of the control group. The primer sequences used in this study were as follows: FOXM1, F: 5'-CCAGCGAACACCACCACATACCG-3', R: 5'-GCGTAATGCTGCTCTAACGCGA-3'; GAPDH: F: 5'- CGCTCTCTGCTCCTCCTGTTC -3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot Analysis

The cells were lysed using 200 μL of radioimmunoprecipitation assay (RIPA) buffer solution containing 1% phenylmethylsulfonyl fluoride (PMSF; Beyotime, Shanghai, China). The protein concentration was detected *via* the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). A proper volume of protein was loaded for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to its concentration. Subsequently, the protein was transferred onto 0.45 mm polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Then, the membrane was washed with Tris-Buffered Saline and Tween-20 (TBST) and sealed in 5% skim milk at 4°C overnight. The primary antibodies were diluted to a suitable concentration using TBST, incubated on a horizontal shaking table at 4°C overnight and washed with TBST for 3 times. Then, the secondary antibodies were diluted with TBST at 1:5000, incubated on the horizontal shaking table at 37°C for 1 h, and washed

with TBST for 3 times. Finally, an appropriate amount of enhanced chemiluminescence (ECL) A solution and B solution (1:1) was prepared into a working solution for band exposure.

MTT Assay

Hep-2 cells were prepared into a cell suspension and seeded into 96-well plates (4×10^3 cells/well). Three duplicate wells were set. After 24 h culture, 20 μL of MTT was added in each well, and cultured again for 4 h. After that, the medium was completely discharged, and 150 μL of dimethyl sulfoxide (DMSO) was added into each well for 15-min incubation at room temperature. The absorbance at the wavelength of 490 nm was measured using a microplate reader.

Colony Formation Assay

The proliferation potential was measured using the colony formation assay. Briefly, the cells (1×10^3 cells/well) were seeded in 6-well plates and incubated at 37°C, in 5% CO_2. Culture medium was changed every 4 days. After 2 weeks, the number of visible colonies was calculated under a microscope.

Invasion and Migration Assay

Matrigel was diluted in serum-free medium (1:8). Next, the mixture was coated on the bottom of the transwell chambers (50 μL/well) and incubated at 37°C for 30 min. Subsequently, 200 μL of cell suspension (serum-free medium) containing 1×10^5 cells were added into the upper chamber, and 500 μL of medium containing 10% FBS was added into the bottom chamber, followed by 24-h culture. On the other day, the transwell chambers were taken out, washed with PBS for 3 times, and fixed with 4% paraformaldehyde. Next, the cells on the surface of the chambers were wiped. The cells penetrating to the bottom were dyed with 0.1% crystal violet staining solution at room temperature for 15 min. Finally, 5 fields of vision were randomly selected under a microscope, followed by observation, photography, and cell counting.

Statistical Analysis

The data were analyzed by GraphPad Prism 7.03 statistical software (La Jolla, CA, USA). The *t*-test was used for analyzing the measurement data. The differences between two groups were analyzed using the Student's *t*-test. Comparison between multiple groups was made using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

FOXM1 Was Upregulated in LC Tissues and Cells

FOXM1 has been demonstrated as a tumor-associated gene[15-17]. To investigate the effect of FOXM1 gene on LC, we used the qRT-PCR assay to detect the expression of FOXM1 in LC tissues and the corresponding adjacent tissues, as well as HaCaT and Hep-2 cells. As shown in Figure 1A, the mRNA level of FOXM1 was significantly upregulated in LC tissues compared with adjacent tissues. Correspondingly, we also detected an upregulated expression of FOXM1 in Hep-2 cells than that in HaCaT cells, as determined by qRT-PCR and Western-blot analysis (Figure 1B-1D).

Knockdown of FOXM1 Reduced Cell Proliferation

To further explore the effect of FOXM1 on the development of LC, we designed two groups: control group (normal Hep-2 cells) and si-FOXM1 group (Hep-2 cells transfected with FOXM1 si-RNA). The results in Figure 2 suggested that the protein expression of FOXM1 in Hep-2 cells was successfully inhibited by FOXM1 si-RNA transfection. Based on this, we performed MTT assay and colony formation assay to detect cell proliferation. We found that cells in the si-FOXM1 group showed a decreased cell proliferation after knockdown of FOXM1, which was indicated by the decreased OD value and cell colony number (Figure 3).

FOXM1 Affected the AKT Signaling Pathway

The PI3K/AKT pathway is widely involved in the development and progression of various kinds of cancers. This pathway triggers nuclear translocation of various cell membrane receptor tyrosine kinases, thus regulating cell processes, including cell proliferation and differentiation[18-20]. As shown in Figure 4A, we found that the p-AKT/total-AKT ratio in the si-FOXM1 group was significantly downregulated compared with the control group. Therefore, we speculated that FOXM1 affected the progression of LC by affecting the phosphorylation of AKT. Sc79, an AKT activator, was applied here to verify our hypothesis[21,22]. We, then, designed three

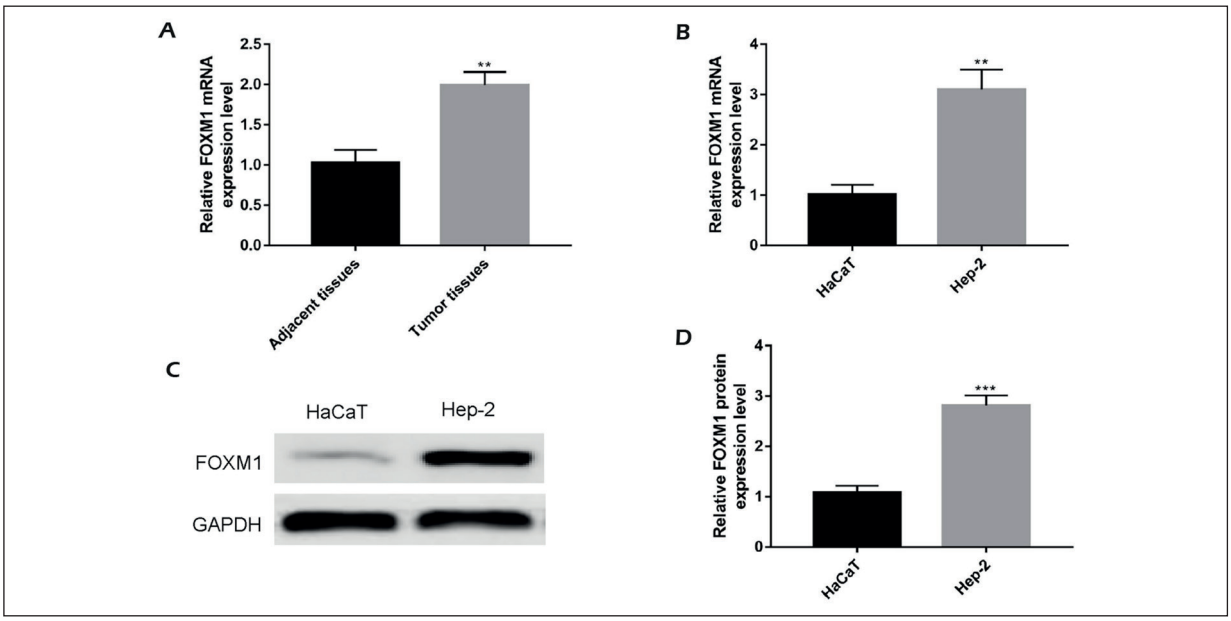

Figure 1. Expression level of FOXM1 in LC tissues and cells. **A,** FOXM1 expression was upregulated in LC tissues compared with corresponding adjacent normal tissues detected by qRT-PCR. **B,** FOXM1 expression was upregulated in Hep-2 cells than HaCaT cells detected by qRT-PCR. **C,** Protein expression of FOXM1 was higher in Hep-2 cells than HaCaT cells. **D,** Qualification of FOXM1 protein expression. (***p*<0.01, *vs.* control group; ****p*<0.001, *vs.* control group).

groups: control group (normal Hep-2 cells), si-FOXM1 group (Hep-2 cells transfected with FOXM1 siRNA), and si-FOXM1+Sc79 (Hep-2 cells transfected with FOXM1 siRNA and treated with Sc79). The results in Figure 4B-4D suggested that the impaired proliferation activ-

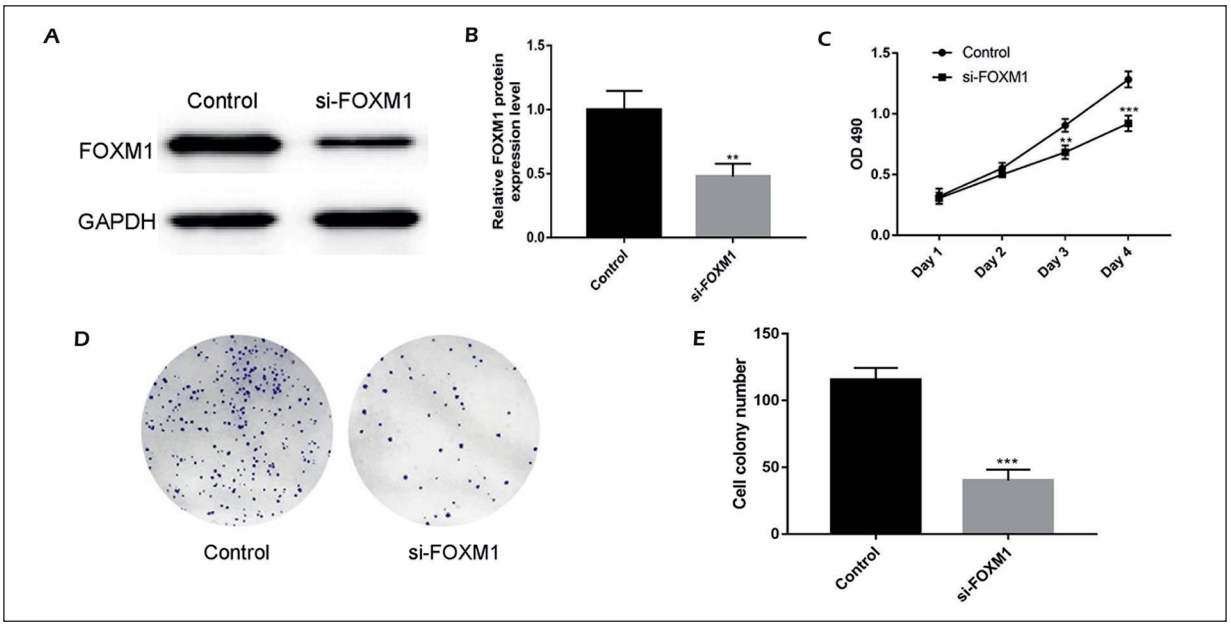

Figure 2. Effects of FOXM1 on the proliferation of Hep-2 cells. **A,** Protein expression of FOXM1 was inhibited after Hep-2 cells were transfected with FOXM1 siRNA. **B,** Qualification of FOXM1 protein expression. **C,** Proliferation ability of Hep-2 cells was impaired after transfection with FOXM1 siRNA detected by MTT assay. **D,** Representative images of colony formation in Hep-2 cells transfected with FOXM1 siRNA or negative control (magnification: 10×). **E,** Cell colony number in control group and si-FOXM1 group. (***p*<0.01, *vs.* control group; ****p*<0.001, *vs.* control group).

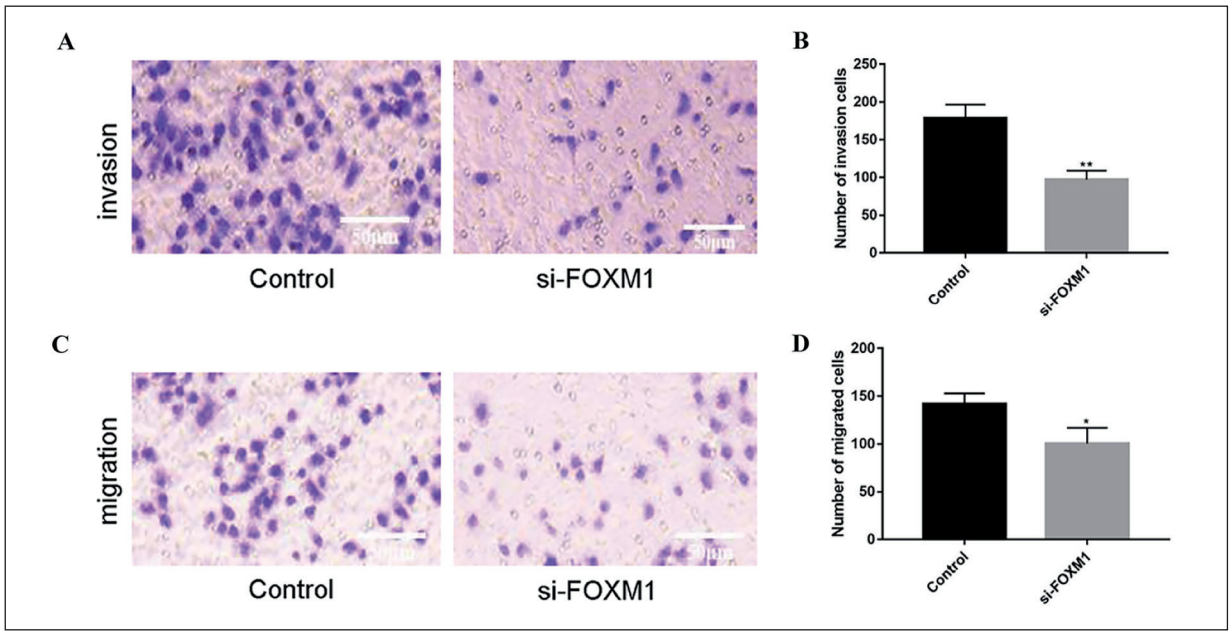

Figure 3. Effects of FOXM1 on the invasion and migration of Hep-2 cells. **A,** Representative images of invasion (**A**) and migration (**C**) assay in Hep-2 cells transfected with FOXM1 siRNA or negative control (magnification: 40x). **B,** Number of invasion cells (**B**) and migrated cells (**D**) in control group and si-FOXM1 group. (**p*<0.05, *vs.* control group; ***p*<0.01, *vs.* control group).

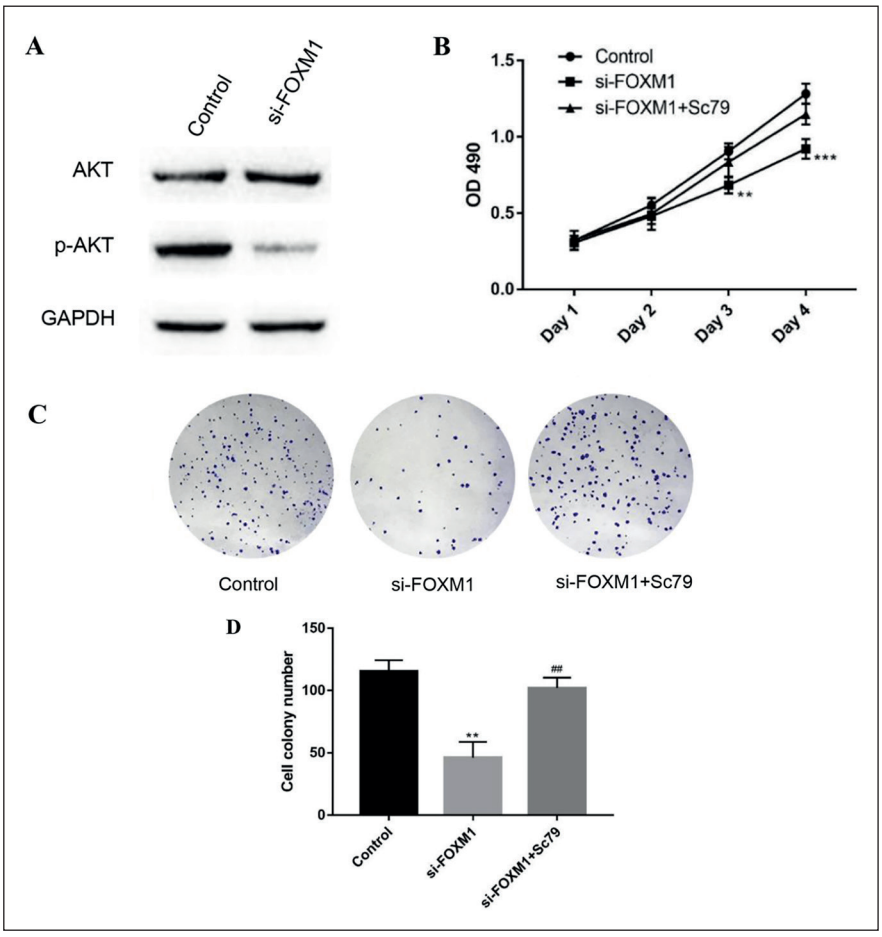

Figure 4. FOXM1 exerted its function through the AKT signaling pathway. **A,** Knockdown of FOXM1 inhibited p-AKT but not AKT expression level. **B-C,** Sc79 recued proliferation ability inhibited by FOXM1 knockdown in Hep-2 cells (magnification × 10). **D,** Cell colony number in control group, si-FOXM1 group, and si-FOXM1+Sc79 group. (***p*<0.01, *vs.* control group; ****p*<0.001, *vs.* control group $^{\#\#}p$<0.01 *vs.* si-FOXM1 group).

ity after transfection of FOXM1 si-RNA could be ameliorated by Sc79. Similarity, attenuated invasion and migration due to FOXM1 knockdown also showed an improvement by treating with Sc79 in Hep-2 cells (Figure 5).

Discussion

LC is one of the most common tumors in the world. Although great progress has been achieved, the long-term prognosis of LC patients is still not satisfactory[1]. Similar to that of other tumors, the complex pathogenesis of LC involves the invasion and adhesion abilities of cancer cells, extracellular matrix degradation, angiogenesis, and chemotaxis[23,24]. In addition, abnormal activation of oncogenes, inactivation of tumor-suppressor genes, and dysregulated signaling pathways exerted crucial effects on the local recurrence, lymph node metastasis, and distant metastasis of LC[25]. Therefore, it is extremely important to search for molecules and signaling pathways that are closely related to LC recurrence and metastasis, so as to provide effective intervention or preventive strategies for LC patients. Belonging to the forkhead transcription factor family, FOXM1 was first discovered in mice by Korver et al[26]. In normal cell growth, the expression of FOXM1 is controlled by two kinds of signaling pathways. The first one stimulates the expression of FOXM1, including MEK1, Ras, and cyclin/CDK, and the other one inhibits the expression of FOXM1, such as RB, ARF, p16, p21, and p27. Under the control of the two mutually antagonistic kinds of signaling pathways, FOXM1 could maintain the normal physiological functions of cells[7]. In addition, FOXM1 in organisms could be acetylated by the p300/CBP pathway, which in turn enhances its DNA binding capacity, transcriptional activity, protein stability, and phosphorylation sensitivity, whereas deacetylated FOXM1 shows opposite effects[6]. FOXM1 has become a widely recognized pro-oncogenic transcription factor, which is upregulated in various solid tumors and closely related to tumor proliferation, invasion, and migration[27,28]. In the present research, we found that the mRNA expression of FOXM1 in LC tissues was significantly higher than that in the corresponding adjacent tissues. Furthermore, in contrast to HaCaT cells, the mRNA and protein levels of FOXM1 were significantly upregulated in Hep-2 cells. These results suggested that FOXM1 was likely to be related to the pathogenesis of LC. Next, we studied the effects of FOXM1 on the cell proliferation of Hep-2 cells. The knockdown of FOXM1 significantly reduced the proliferation of Hep-2 cells. The PI3K/AKT signaling pathway is widely studied in the development and progression of various cancers,

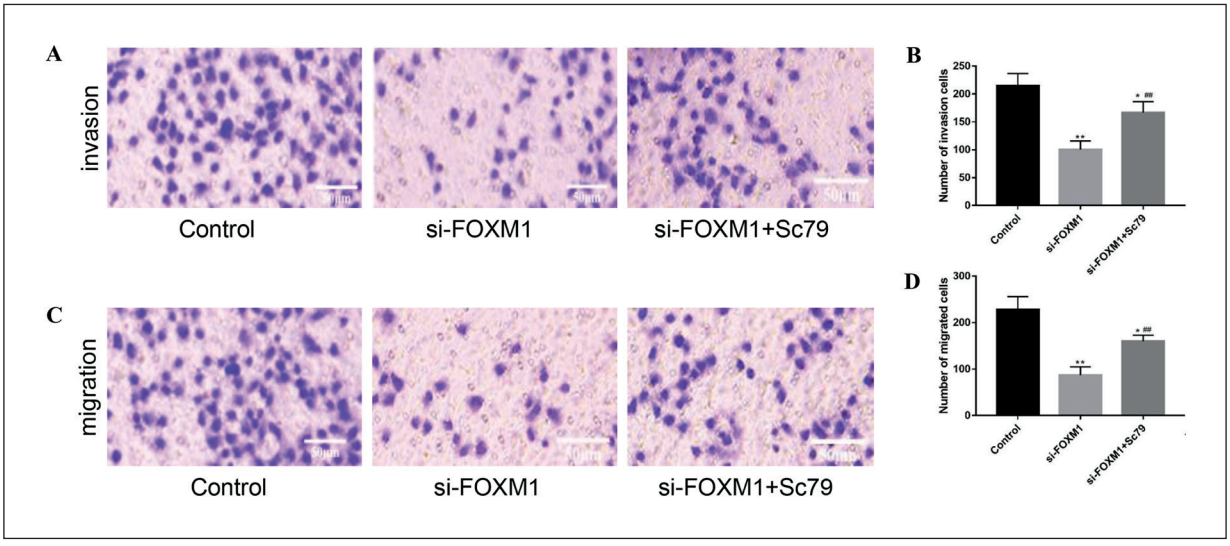

Figure 5. Activation of AKT signaling pathway rescued the invasion and migration of Hep-2 cells. **A,** Sc79 reversed the inhibited invasion in Hep-2 cells induced by FOXM1 knockdown (magnification × 40). **B,** Number of invasion cells in control group, si-FOXM1 group and si-FOXM1+Sc79 group. **C,** Sc79 reversed the inhibited migration in Hep-2 cells induced by FOXM1 knockdown (magnification × 40). **D,** Number of migratory cells in control group, si-FOXM1 group and si-FOXM1+Sc79 group. (*p<0.05, *vs.* control group; **p<0.01, *vs.* control group; $^{\#\#}p$<0.01 *vs.* si-FOXM1 group).

which exerts an important role in cell proliferation, differentiation, and apoptosis. So, Han et al[29] observed that the knockdown of DUXAP10 could attenuate the proliferation and metastasis of hepatocellular carcinoma cells by regulating the PI3K/Akt signaling pathway. Kachrilas et al[30] reported that the activated PI3K/AKT pathway contributes to stimulate the progression of bladder cancer. In this paper, we detected the downregulated p-AKT/total AKT ratio in Hep-2 cells transfected with FOXM1 si-RNA compared to untreated Hep-2 cells. Based on this, we speculated that FOXM1 potentially exerted its biological function by activating the AKT signaling pathway. To detect this, Sc79, a powerful AKT activator was applied in Hep-2 cells. To our surprise, the damaged cell proliferation, invasion, and migration capacities after the knockdown of FOXM1 were partially restored by Sc79 treatment, indicating that FOXM1 regulated the metabolism of Hep-2 cells by activating the AKT signaling pathway.

Conclusions

We revealed that FOXM1 was highly associated with cell proliferation, invasion, and migration of LC cells. The AKT signaling pathway might be the downstream target of FOXM1. Thus, our results revealed that FOXM1 might serve as a potential diagnostic and therapeutic molecular marker LC treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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